The Association of Flap Endonuclease 1 Genotypes with the Risk of Childhood Leukemia

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Abstract. *Aim: Flap endonuclease 1 (FEN1) is one of the most important proteins in maintaining genome stability and preventing carcinogenesis. In recent years, the contribution of two variants of FEN1, rs174538 and rs4246215, regarding cancer risk have been investigated in lung, breast, liver, esophageal, gastric, colorectal cancer and glioma. However, it has not been revealed whether rs174538 and rs4246215 are associated with leukemia. Therefore, in the present study we aimed to evaluate the contribution of these genotypic polymorphisms in FEN1 to childhood acute lymphoblastic leukemia (ALL) risk in Taiwan. Materials and Methods: In total, 266 patients with childhood ALL and an equal number of recruited non-cancer controls were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Results: The FEN1 rs174538 genotype, but not rs4246215, was differently distributed between childhood ALL and control groups. The AG and AA of FEN1 rs174538 genotypes were significantly less frequently found in childhood ALL patients than in controls (odds ratio [OR]=0.68 and 0.48, 95%confidence intervals [CI]=0.47-0.98 and 0.24-0.82, respectively). As for gender, boys carrying the FEN1 rs174538 AG or AA genotype conferred lower ORs of 0.55 and 0.36 (95%CI=0.33-0.91 and 0.18-0.73, p=0.0053) for childhood ALL. Regarding age, those equal to or greater than 3.5 years of age at onset carrying the FEN1 rs174538 AG or AA genotype were of lower risk (ORs=0.53 and 0.32, 95%CI=0.31-0.90 and 0.15-0.70, p=0.0042). Conclusion: The FEN1 rs174538 A allele is a protective biomarker for childhood ALL and this association is more significant in males and in patients at onset age of 3.5 years or older.*

Acute lymphoblastic leukemia (ALL) is the most common pediatric leukemia, accounting for 25-30% of all childhood malignancies (1). Worldwide, the annual incidence rate of childhood ALL is approximately ten cases per 100,000, with peak incidence occurring at the age of 2 to 5 years (2). While the clinical, pathological and immunophenotypic features of ALL are well-documented, its etiology has not been fully clarified (1). In the literature, several environmental factors, such as ionizing radiation, parental use of alcohol and tobacco, and virus exposure, have been identified as potential risk factors for the development of childhood ALL. But among them, only ionizing radiation has been confirmed (3). Recently, mounting evidence suggest that genetic factors play a significant role in the development of childhood ALL. For instance, inherited genetic disorders, such as Down syndrome and Fanconi anemia, have been associated with an enhanced ALL risk (4, 5). Additionally, genetic mutations in several cancer-related genes, such as *p53*, *N-ras*, and *PHF6*, have frequently been identified in ALL patients (6); and finally, only a small fraction of children who are exposed to environmental factors go on to develop ALL, indicating the potential for a genetic predisposition to develop childhood ALL (1).

It is believed that DNA repair genes are encoding enzymes closely-related to maintainance of genomic stability and prevention of tumorigenesis. Among these genes, Flap endonuclease 1 (*FEN1*) is a multiple-function endonuclease, playing a role in both base excision repair (BER) and DNA replication (7). In the BER pathway, FEN1 could efficiently remove 5' flap (8, 9). During the DNA replication process,

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Table I. *Demographic data of 266 childhood ALL patients and 266 matched controls.*

Characteristic	Controls (n=266)			Patients (n=266)			*p*-Value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			8.3 (4.8)			7.0 (4.4)	0.64
Gender							1.00
Boys	148	55.6%		148	55.6%		
Girls	118	44.4%		118	44.4%		

[a]Based on Chi-square test.

FEN1 could promote the maturation of Okazaki fragments (8, 9). Additionally, FEN1 could also act as a 5' exonuclease (10) and a gap-dependent endonuclease (11, 12), which could promote apoptotic DNA fragmentation during apoptotic processes. Elevated rate of spontaneous mutations are observed in cells with functionally impaired yeast FEN1 (known as RAD27 in yeast) (12-14). In mouse models, loss of function of FEN1 can lead to increased genomic instability and carcinogenesis (15). In human cancer cells, *FEN1* mutations resulting in reduced nuclease activity have been found, and about 70% of mice knocked-in with the mutated *FEN1* have developed tumors in multiple organs (16). Therefore, dysregulated expression of FEN1 resulting from genetic variations may contribute to susceptibility to all types of cancers.

Previous investigations of *FEN1* genomic variations focused on the two polymorphisms, promoter -69G>A (rs174538) and 3'UTR 4150G>T (rs4246215), both were effective on influencing the expression level of FEN1 and enzyme activity in transfected cells (17). In the literature, the polymorphic genotypes of rs174538 and/or rs4246215 are associated with the risk of lung cancer (17), gastrointestinal cancer (18), esophageal cancer (19), breast cancer (20) and glioma (21). However, the contribution of *FEN1* genomic variation to leukemia has never been investigated.

The purpose of the present study was, therefore, to analyze the genetic polymorphisms of *FEN1* promoter -69G>A (rs174538) and 3'UTR 4150G>T (rs4246215) in a representative pediatric population sample (control/case= 266/266), to investigate the correlation between *FEN1* genotypes and childhood ALL in Taiwanese children.

Materials and Methods

Study population and sample collection. The Institutional Review Board of the China Medical University Hospital approved our study, and written informed consent was obtained from one or both parents of participants. Two hundred and sixty-six patients diagnosed with childhood ALL (all patients under 18 years of age) were recruited between 2005-2010 from the general surgery outpatient clinics within the Pediatric Departments at China Medical University Hospital and the National Taiwan University Hospital, Taiwan, Republic of China. Clinical characteristics of patients, including their histological details, were identified by expert surgeons. All subjects voluntarily participated, completed a questionnaire with the help of parents or guardians and provided peripheral blood samples. The questionnaire recorded their disease history, diet and sleep lifestyles and the disease history, diet and behavioral lifestyle, social-economic status of the parents. An equal number of age-matched non-cancer healthy volunteers were selected for use as a control group following initial random sampling from the Health Examination Cohort established from 2005 to 2010 as previously published (22, 23). The registered health practitioners in the hospital provided a multidisciplinary team approach of health assessment for the volunteers. Most of the volunteers underwent health examinations every 5 to 6 months. A total of 457 volunteers aged under 18 years were recruited into the study. They were cancer-free by the age of diagnosis according to International Classification of Disease, ninth revision (ICD-9) codes. Finally, 266 participants were included for analysis in the study since we had to match the population structure (number, age and gender) with our case population. The overall agreement rate in the study was above 85%.

Genotyping assays. Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan), stored at –80˚C, diluted and aliquotted for genotyping as working stock at –20˚C (24, 25). Genotyping for *FEN1* rs174538 and rs4246215 of all subjects was carried-out by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assays, as previously reported (26, 27). The primers were designed by Terry Fox Cancer Research team and the PCR-RFLP conditions for *FEN1* rs174538 and rs4246215 were summarized in Table II. The success rate of PCR-RFLP was 100%, and the genotypes of 5% of the participants in both the control and patient groups were analyzed by PCR direct sequencing (Genomics BioSci & Tech Co., Taipei). The consistency between direct sequencing and PCR-RFLP was 100%.

Statistical analysis. Participants having both genotypic and clinical data (control/case=266/266) were selected for the final analysis. The descriptive statistics of patients and controls were presented as the mean±standard deviations (SDs) or as percentages. The Pearson's Chi-square test or Fisher's exact test (when any cell was less than five) was used to compare the distribution of the genotypes. Associations were expressed and evaluated as odds ratios (ORs) with 95% confidence intervals (95%CIs). Statistical tests were deemed significant when the *p*-value was less than 0.05.

Table II. *Summary of the rs numbers, primers, amplicon lengths before and after enzyme digestion, restriction enzymes for FEN1 rs174538 and rs4246215.*

rs number	Primer sequence	Restriction enzyme	Amplicon length	Genotypes and enzymatic fragment sizes
rs174538	F: 5'-CCTAAGGAGTTCATGGCAAG-3'	*Sal* I	307 bp	G: 307 bp
	R: 5'-AATCGCAGGACTACAAGTCC-3'			A: 223 + 84 bp
rs4246215	F: 5'-GGTGGAGAGAGGATTCTAAG-3'	*BcoD* I	382 bp	T: 382 bp
	R: 5'-CATCTGCTAAGATGCGCCTT-3'			G: 234 + 148 bp

Table III. *Distribution of the FEN1 genotypic and allelic frequencies among 266 childhood ALL patients and 266 healthy controls.*

Genotype	Controls	%	Cases	%	*p*-Value[a]	OR (95% CI)[b]
Promoter rs174538						
GG	102	38.4%	133	50.0%	0.0115*	1.00 (Reference)
AG	119	44.7%	105	39.5%		0.68 (0.47-0.98)*
AA	45	16.9%	28	10.5%		0.48 (0.24-0.82)*
GA+AA	164	61.6%	133	50.0%		0.62 (0.44-0.88)*
G allele	323	60.7%	371	69.7%	0.0020*	1.00 (Reference)
A allele	209	39.3%	161	30.3%		0.67 (0.52-0.86)*
3' UTR rs4246215						
GG	99	37.2%	92	34.6%	0.7933	1.00 (Reference)
GT	122	45.9%	125	47.0%		1.10 (0.76-1.61)
TT	45	16.9%	49	18.4%		1.17 (0.71-1.92)
GT+TT	167	62.8%	174	65.4%		1.12 (0.79-1.60)
G allele	320	60.2%	309	58.1%	0.4927	1.00 (Reference)
T allele	212	39.8%	223	41.9%		1.09 (0.85-1.39)

[a]Based on Pearson's chi-square test; [b]OR: odds ratio; CI: confidence interval. *Statistically significant.

Results

The frequency distributions for the age and gender of 266 childhood ALL patients and 266 non-cancer healthy controls are shown in Table I. The characteristics of patients and controls were well-matched ($p>0.05$) (Table I).

The genotypic and allelic frequencies for the *FEN1* rs174538 and rs4246215 among the controls and childhood ALL patients are shown in Table III. The genotypic frequency distributions for *FEN1* rs174538 were significantly different between childhood ALL and control groups ($p=0.0115$), while those for *FEN1* rs4246215 polymorphism were not significantly different ($p>0.05$) (Table III). Those who carried AG, AA, AG plus AA genotypes had a significantly reduced risk of ALL with ORs of 0.68, 0.48, and 0.62 respectively compared to those with the wild-type GG genotype (95%CI=0.47-0.98, 0.24-0.82 and 0.44-0.88, respectively). From the results of allelic frequency analysis, we can find that the A allele of *FEN1* rs174538 seemed to be a protective factor for childhood ALL ($p=0.0020$, OR=0.67, 95%CI=0.52-0.86), while the T allele of *FEN1* rs4246215 was not ($p=0.4927$, OR=1.09, 95%CI=0.85-1.39). The conclusion that can be deduced from Table III is that the *FEN1* AG and AA genotypes (A allele) seemed to be a protective factor for childhood ALL in Taiwan.

Because age and gender are predominant risk factors for developing childhood ALL, the contribution of the *FEN1* genotype to childhood ALL stratified by age and gender were further analyzed and presented in Table IV. The average age of onset for the 133rd and 134th subjects in the control and patient groups was 3.5 years; thus, we further stratified the groups into <3.5 and ≥3.5 year-old sub-groups. Noticeably, in the elder-onset (≥3.5 years) group, subjects with hetero-variant AG or homo-variant AA genotypes for *FEN1* rs174538 had lower risks for developing childhood ALL than those with the wild-type GG genotype (p for trend=0.0042, OR=0.53 and 0.32, CI=0.31-0.90 and 0.15-0.70 for AG and AA, respectively), the combined AG with AA group also has the similar results (OR=0.47, CI=0.29-0.76); however, this was not the case for the younger (<3.5 years) group (Table IV). As for gender, boys with AG or GG genotypes for *FEN1* rs174538 were less likely to develop childhood ALL than those with the homozygous GG

Table IV. *Distribution of the FEN1 promoter rs174538 and 3' UTR rs4246215 genotypes stratified by age and gender*

Characteristics	Promoter rs174538				3' UTR rs4246215			
	Controls n (%)	Cases n (%)	P_{trend}[a]	OR (95% CI)[b]	Controls n (%)	Cases n (%)	P_{trend}[a]	OR (95% CI)[b]
Onset age								
<3.5 years			0.6886				0.9127	
Wild-type	52 (39.10)	58 (43.61)		1.00 (Reference)	51 (38.35)	48 (36.09)		1.00 (Reference)
Hetero-variant	61 (45.86)	59 (44.36)		0.87 (0.52-1.46)	59 (44.36)	60 (45.11)		1.08 (0.63-1.84)
Homo-variant	20 (15.04)	16 (12.03)		0.72 (0.34-1.53)	23 (17.29)	25 (18.80)		1.15 (0.58-2.30)
Hetero- + homo-variants	81 (60.90)	75 (56.39)		0.83 (0.51-1.35)	82 (61.65)	85 (63.91)		1.10 (0.67-1.81)
≥3.5 years			0.0042*				0.8641	
Wild-type	50 (37.59)	75 (56.39)		1.00 (Reference)	48 (36.09)	44 (33.08)		1.00 (Reference)
Hetero-variant	58 (43.61)	46 (34.59)		0.53 (0.31-0.90)*	63 (47.37)	65 (48.87)		1.13 (0.66-1.92)
Homo-variant	25 (18.80)	12 (9.02)		0.32 (0.15-0.70)*	22 (16.54)	24 (18.05)		1.19 (0.59-2.42)
Hetero- + homo-variants	83 (62.41)	58 (43.61)		0.47 (0.29-0.76)*	85 (63.91)	89 (66.92)		1.14 (0.69-1.89)
Gender								
Boys			0.0053*				0.8793	
Wild-type	55 (37.16)	81 (54.73)		1.00 (Reference)	53 (35.81)	49 (33.11)		1.00 (Reference)
Hetero-variant	63 (42.57)	51 (34.46)		0.55 (0.33-0.91)*	68 (45.95)	70 (47.30)		1.11 (0.67-1.86)
Homo-variant	30 (20.27)	16 (10.81)		0.36 (0.18-0.73)*	27 (18.24)	29 (19.59)		1.16 (0.61-2.23)
Hetero- + homo-variants	93 (62.84)	67 (45.27)		0.64 (0.40-0.99)*	93 (64.19)	99 (66.89)		1.15 (0.71-1.86)
Girls			0.7326				0.8978	
Wild-type	47 (39.83)	52 (44.07)		1.00 (Reference)	46 (38.98)	43 (36.44)		1.00 (Reference)
Hetero-variant	56 (47.46)	54 (45.76)		0.87 (0.51-1.50)	54 (45.76)	55 (46.61)		1.09 (0.62-1.91)
Homo-variant	15 (12.71)	12 (10.17)		0.72 (0.31-1.70)	18 (15.26)	20 (16.95)		1.19 (0.56-2.54)
Hetero- + homo-variants	71 (60.17)	66 (55.93)		0.84 (0.50-1.41)	72 (61.02)	75 (63.56)		1.11 (0.66-1.89)

[a]P for trend based on Chi-square test. [b]OR, odds ratio; CI, confidence interval. *Statistically significant.

genotype (p for trend=0.0053, OR=0.55 and 0.36, CI=0.33-0.91 and 0.18-0.73 for AG and AA, respectively), but this was not the case for the girls (Table III). As for the *FEN1* rs4246215, there is no difference between the comparisons of stratified age or gender groups. In summary, analyses revealed an interaction between the age of onset and gender among *FEN1* rs174538 genotypes in childhood ALL susceptibility.

Discussion

In the current study, we firstly examined the contribution of *FEN1* genotypes to childhood ALL susceptibility. Since FEN1 plays an essential role in BER, DNA replication, and cell apoptosis, it is very possible that the hereditary genomic variations may determine the personal susceptibility to carcinogenesis. In the literature, the polymorphic *FEN1* genotypes may determine FEN1 enzyme activity and the rate of tumorigenesis in cell and mice models (15, 16), that may be linked to the risk of all types of cancers, including childhood ALL. In the current study, we found that the A variant genotypes of *FEN1* rs174538 were significantly associated with a lower susceptibility to childhood ALL (Table III). These findings are consistent with previous studies that identified the A allele to be a protective factor in several other types of cancer, including lung (17), gastrointestinal (18), esophageal (19), breast cancer (20) and glioma (21). As far as we are concerned, the current study was the first to reveal the genotypic contribution of *FEN1* genotypes to (childhood) ALL, with the positive results for *FEN1* rs174538 and negative for *FEN1* rs4246215 (Table III).

We further analyzed the relationship between the *FEN1* rs174538 genotype and childhood ALL risk according to onset age and gender of the investigated subjects. Interestingly, children ≥3.5 years old with a AG or AA genotype had a lower risk of childhood ALL than those with the wild-type GG genotype. However, this relationship was not found for the group of children <3.5 years old at onset age of childhood ALL (Table IV). Additionally, no such age difference was observed in analyses of the *FEN1* rs4246215 genotype. The mechanisms of FEN1 protein involved in the etiopathology of and progression of ALL may be different for adults and children. Further investigation in adult ALL was needed, to extend the value of our findings to ALL in general. Although no statistical significance was found in the girls-only analysis in Table IV, we could not exclude the possibility that in thousand-level of population pools of ALL patients, there will be a finding as for the girls similar to that

observed for the boys in Table IV for the trend is much similar, but the numbers of homo-variant AA genotypes are somewhat small. The enlarged sample size may provide us with a more realistic answer. Thus, regarding the gender difference in contribution of *FEN1* genotypes to ALL susceptibility between boys and girls, further investigations are required. In the present article, we can conclude that the protective impact of the A allele at *FEN1* rs174538 with respect to ALL risk was more obvious for boys than the girls (Table IV). From the epidemiological viewpoint, the incidence of pediatric hematological malignancies worldwide has increased for boys but not for girls (28, 29). While the complete underlying mechanisms have not been discovered, sexual hormones and steroids reportedly play an important part in controlling the proliferation of leukemic cells. For instance in supporting the idea of gender differences in susceptibility to childhood ALL, 17-β estrogen was found to conduct a stronger inhibitory effect than testosterone on human monoblastic U937 cells (30). At the molecular level, further measurement and analysis of the BER DNA repair capacity among children of different *FEN1* genotypes, ages, and genders may help us further understand the interaction and ALL carcinogenesis.

In conclusion, the present study documented evidence of a positive association between the genotypes of *FEN1* and childhood ALL and examined the age- and gender-interactions with the genotype, in order to determine childhood ALL susceptibility. The presence of the A allele of rs174538 was not only a novel detectable and predictive biomarker for childhood ALL but a protective determinant for boys and patients with onset age of equal to or older than 3.5 years old.

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